A Divergence in the MAP Kinase Regulatory Network Defined by MEK Kinase and Raf

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Mitogen-activated protein kinases (MAPKs) are rapidly phosphorylated and activated in response to various extracellular stimuli in many different cell types. Such regulation of MAPK results from sequential activation of a series of protein kinases. The kinases that phosphorylate MAPKs, the MAP kinase kinases (MEKs) are also activated by phosphorylation. MEKs are related in sequence to the yeast protein kinases Byr1 (from *Schizosaccharomyces pombe*) and Ste7 (from *Saccharomyces cerevisiae*), which function in the pheromone-induced signaling pathway that results in mating. Byr1 and Ste7 are in turn regulated by the protein kinases Byr2 and Ste11. The amino acid sequence of the mouse homolog of Byr2 and Ste11, denoted MEKK (MEK kinase), was elucidated from a complementary DNA sequence encoding a protein of 672 amino acid residues (73 kilodaltons). MEKK was expressed in all mouse tissues tested, and it phosphorylated and activated MEK. Phosphorylation and activation of MEK by MEKK was independent of Raf, a growth factor–regulated protein kinase that also phosphorylates MEK. Thus, MEKK and Raf converge at MEK in the protein kinase network mediating the activation of MAPKs by hormones, growth factors, and neurotransmitters.

Mitogen-activated protein kinases (MAPKs) (also called extracellular signal–regulated kinases or ERKs) are rapidly activated in response to ligand binding by both growth factor receptors that are tyrosine kinases [such as the epidermal growth factor (EGF) receptor] and receptors that are coupled to heterotrimeric guanine nucleotide binding proteins (G proteins) such as the thrombin receptor (*1–3*). The MAPKs appear to integrate multiple intracellular signals transmitted by various second messengers. MAPKs phosphorylate and regulate the activity of enzymes and transcription factors including the EGF receptor (*4*), Rsk 90 (*5*), phospholipase A_2 (*6*), c-Myc (*7*), and perhaps c-Jun (*8*). Although the rapid activation of MAPKs by receptors that are tyrosine kinases is dependent on Ras (*9, 10*), G protein–mediated activation of MAPK appears to occur predominantly through pathways independent of Ras (*1, 11, 12*).

Complementation analysis of the pheromone-induced signaling pathway in yeast has defined a protein kinase system that controls the activity of Spk1 and Fus3-Kss1, the *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* homologs of MAPK (*13–16*). In *S. cerevisiae*, the protein kinase Ste7 is the upstream regulator of Fus3-Kss1 activity; the protein kinase Ste11 regulates Ste7 (*13, 14*). The *S. pombe* gene products Byr1 and Byr2 are homologous to Ste7 and Ste11, respectively (*15, 16*). The similarity of these protein kinase systems to those that regulate MAPKs in mammalian cells has recently been substantiated with the identification of the rodent and human homologs of Ste7 and Byr1 (*17, 18*). The MEK [MAP kinase (or ERK) *k*inase] or MKK (MAP *k*inase *k*inase) enzymes are similar in sequence to Ste7 and Byr1. The MEKs phosphorylate MAPKs on both tyrosine and threonine residues, which results in activation of MAPK (*19, 20*). The mammalian serine-threonine protein kinase Raf phosphorylates and activates MEK (*21, 22*), which leads to activation of MAPK. Raf is activated in response to growth factor receptor tyrosine kinase activity (*23*); therefore Raf may activate MAPK in response to stimulation of membrane-associated tyrosine kinases. Raf is unrelated in sequence to Ste11 and Byr2. Thus, Raf may represent a divergence in mammalian cells from the pheromone-responsive protein kinase system defined in yeast. Cell- and receptor-specific differences in the regulation of MAPKs suggest that other Raf-independent regulators of mammalian MEKs exist (*3, 4, 11, 12*).

Sequences that are identical in Ste11 and Byr2 were used to identify the mammalian MEK kinase (MEKK) cDNA. Degenerate oligodeoxynucleotides were designed to correspond to regions of sequence identity between the *STE11* and *byr2* genes (*15, 24*). With these primers and cDNA templates derived from polyadenylated RNA from NIH 3T3 cells, a polymerase chain reaction product of 320 base pairs (bp) was isolated and found to be similar in sequence to both *STE11* and *byr2*. This 320-bp cDNA was used as a probe to obtain a MEKK cDNA of 3260 bp from a mouse brain cDNA library. On the basis of the Kozak consensus sequence for initiation codons (*25*), the starting methionine can be predicted to occur at nucleotide 486. With this methionine at the start, the cDNA encodes a protein of 672 amino acids, corresponding to a molecular size of 73 kD (Fig. 1A). There is another in-frame methionine at position 441, which does not follow the Kozak rule, but would yield a protein of 687 amino acid residues (74.6 kD). This size range correlates with the apparent molecular size of 78 to 80 kD of MEKK determined by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

The primary sequence of the MEKK protein suggests two functional domains, an NH_2-terminal moiety rich in serine and threonine that may serve a regulatory role and a COOH-terminal protein kinase catalytic domain (Fig. 1A). Twenty percent of the NH_2-terminal 400 amino acids are serine or threonine whereas there are only two tyrosines. Several potential sites of phosphorylation by protein kinase C are apparent in the NH_2-terminal region; no SH2 or SH3 domains are encoded in the MEKK sequence. The catalytic domain is located in the COOH-terminal half of MEKK and contains consensus sequences corresponding to protein kinase subdomains I to XI (*26*). The catalytic domain shows approximately 75 percent similarity and 35 percent identity with the amino acid sequences of the catalytic domains of Byr2 and Ste11 (Fig. 1B). Ste11 contains an insert between kinase subdomains II and III that is not present in Byr2 or MEKK. The NH_2-terminal moieties of MEKK, Ste11, and Byr2 show little similarity although the three kinases are of similar size.

MEKK is encoded by a 7.8-kb mRNA that is expressed in several cell lines and mouse tissues (Fig. 2A) and appears to be the product of a single gene (Fig. 2B). The MEKK mRNA is highly expressed in mouse heart and spleen, whereas low amounts are present in liver. The 7.8-kb MEKK mRNA was identified with probes derived from both the 5′ and 3′ ends of the MEKK cDNA. Thus, the MEKK cDNA is missing a putative untranslated sequence of about 4 kb. Immunoblots of cell lysates probed with

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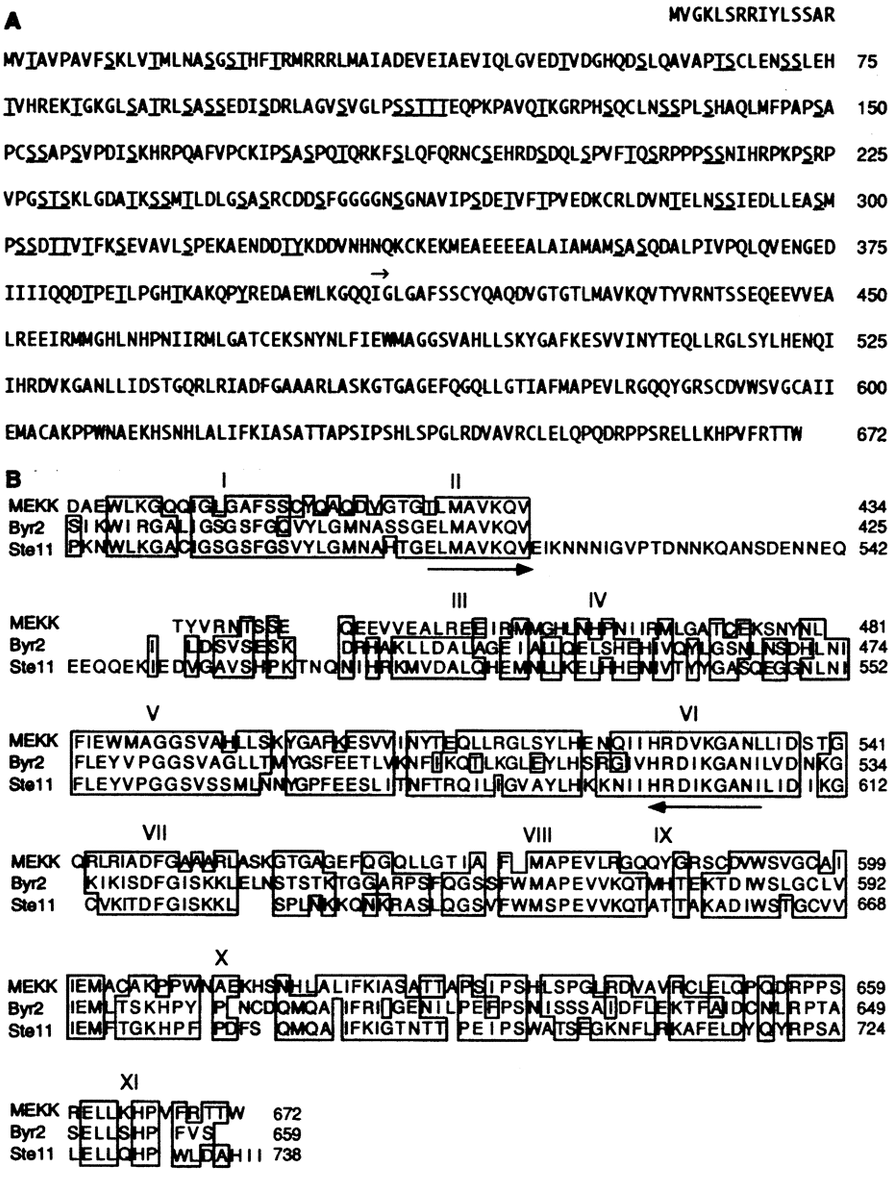

affinity-purified antibodies to the 15–amino acid peptide DRPPSRELLKHPVFR derived from the COOH-terminus of MEKK defined a prominent band migrating at 78 kD (Fig. 2C). Pheochromocytoma (PC12), Rat 1a, and NIH 3T3 cells contained the same 78-kD immunoreactive protein, which often migrated as a doublet on SDS-PAGE. A prominent 50-kD immunoreactive spe-

Fig. 1. Amino acid sequence of MEKK and comparison to the kinase domains of Byr2 and Ste11. (**A**) The predicted amino acid sequence of the MEKK cDNA. The MEKK nucleotide sequence was determined by dideoxynucleotide sequencing of double-stranded DNA and reported to the GenBank database (accession number L13103). The NH_2-terminal 15 amino acids represent additional sequence encoded by a second potential start site upstream of the Kozak consensus sequence; the starting methionine is designated as amino acid number 1. Serine, threonine, and tyrosine residues in the NH_2-terminal moiety are underlined. The catalytic domain is indicated by an arrow. (**B**) Alignment of the MEKK catalytic domain with the catalytic domains of Byr2 and Ste11. Boxed regions indicate amino acid sequence similarity or identity. Consensus sequences corresponding to conserved kinase subdomains I to XI (*26*) are indicated by roman numerals. Arrows indicate the locations in the amino acid sequence that correspond to the sense [GA(AorG)(CorT)-TIATGGCIGTIAA(AorG)CA] and antisense [TTIGCICC(TorC)TTIAT(AorG)TCIC(GorT)-(AorG)TG] degenerate oligodeoxynucleotides used to prime cDNA templates derived from NIH 3T3 cells in the polymerase chain reaction. The groups of amino acids considered to be similar were Cys; Ser, Thr, Pro, Ala, and Gly; Asn, Glu, Asp, and Gln; His, Arg, and Lys; Met, Ile, Leu, and Val; Phe, Tyr, and Trp. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

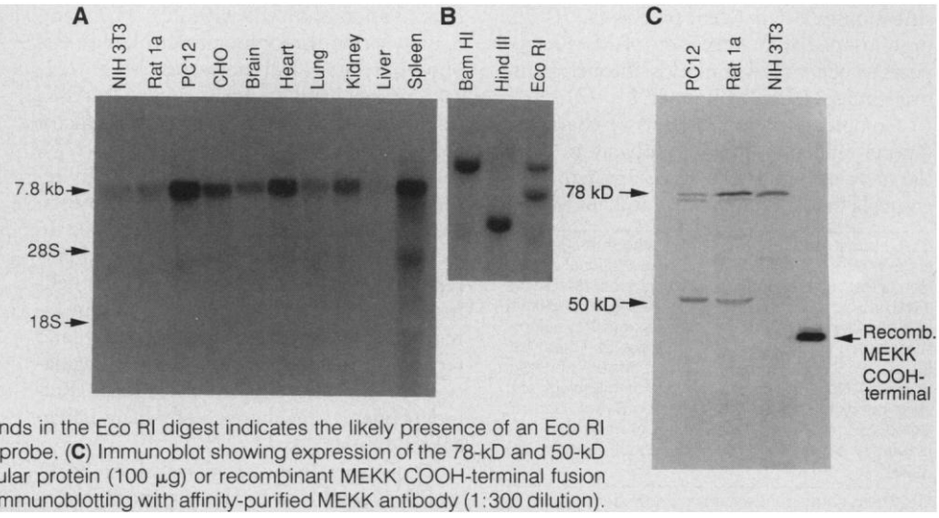

Fig. 2. Expression of MEKK gene products. (**A**) Northern (RNA) blot of a single 7.8-kb MEKK mRNA in several cell lines and mouse tissues. Equal amounts (20 μg) of total RNA were loaded onto the gel as indicated by ethidium bromide staining. Blots were probed with either a 320-bp cDNA fragment encoding a portion of the MEKK kinase domain or a 858-bp fragment encoding a portion of the NH_2-terminal region of MEKK. (**B**) Southern (DNA) blot of MEKK gene. Mouse genomic DNA (10 μg; Clonetech) was digested with either Bam HI, Hind III, or Eco RI and applied to gels. Blots were probed with a 320-bp fragment of the MEKK gene. The appearance of one band in the Bam HI and Hind III digests suggests that MEKK is encoded by one gene. The appearance of two bands in the Eco RI digest indicates the likely presence of an Eco RI site within an intronic sequence spanned by the probe. (**C**) Immunoblot showing expression of the 78-kD and 50-kD forms of MEKK in rodent cell lines. Soluble cellular protein (100 μg) or recombinant MEKK COOH-terminal fusion protein (30 ng) (*31*) was loaded onto the gel for immunoblotting with affinity-purified MEKK antibody (1:300 dilution).

cies was also commonly present, but varied in intensity from preparation to preparation; hence, it may be a proteolytic fragment of the 78-kD protein. Visualization of both the 78- and 50-kD immunoreactive bands on immunoblots was inhibited by incubation of the 15–amino acid peptide antigen with the antibody (*27*). The MEKK protein detected by immunoblotting is similar to the molecular size predicted from the open reading frame of the MEKK cDNA.

The MEKK protein was expressed in COS-1 cells to define its function in regulating the signaling system that includes MAPK. When MEKK was overexpressed in COS-1 cells, MAPK activity was four to five times greater than that in control cells transfected with plasmid lacking a MEKK cDNA insert (Fig. 3A). The activation of MAPK occurred in COS cells deprived of serum and in the absence of any added growth factor. The activity of MAPK was similar to that observed after stimulation of control cells with EGF. Stimulation of COS cells transiently overexpressing MEKK with EGF resulted in only a slight increase in MAPK activity compared to that observed with MEKK expression alone. The MEKK protein was detected in transfected COS cells by immunoblotting (Fig. 3B). Only the 50-kD MEKK immunoreactive fragment was detected in lysates from control COS cells. Transient expression of MEKK in COS cells yielded a predominant 82-kD band that was slightly larger than that observed in PC12, Rat1a, or NIH 3T3 cells. This apparently results from the use of the methionine at position 441 rather than 486 for initiation of translation. The bands above the 82-kD MEKK band appear to result from phosphorylation of the MEKK protein; the group of bands below the 82-kD MEKK protein may result from proteolysis. Addition of the 15–amino acid MEKK peptide antigen to the antiserum during immunoblotting prevented detection of all of the immunoreactive bands (*27*); these bands were not detected in extracts of control COS cells, an indication that they were derived from the expressed MEKK protein.

Expression of MEKK activated MEK, the kinase that phosphorylates and activates MAPK (Fig. 4A). We used recombinant MAPK to assay MEK activity (*28*) in COS cell lysates that had been fractionated by fast protein liquid chromatography (FPLC) on a Mono S column. In this assay, the ability of each column fraction to activate added recombinant MAPK was mea-

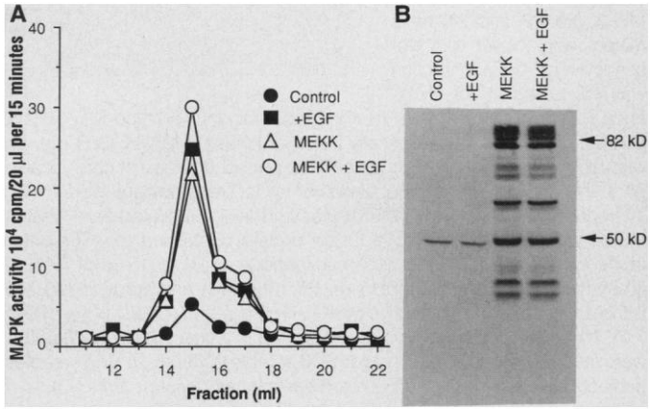

Fig. 3. Activation of MAPK in COS cells transfected with MEKK. (**A**) COS cells in 100-mm culture dishes were transfected as described (*36*) with either the pCMV5 expression vector alone (1 μg; control) or the pCMV5-MEKK construct (1 μg; MEKK). After 48 hours, the cells were placed in serum-free medium containing bovine serum albumin (0.1 percent) for 16 to 18 hours to induce quiescence. Cells were then treated with human EGF (30 ng/ml) (+EGF) or buffer (control) for 10 minutes, washed twice in cold phosphate-buffered saline (PBS), and lysed in cell lysis buffer containing 50 mM β-glycerophosphate (pH 7.2), 100 μM sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, Triton X-100 (0.5 percent), leupeptin (2 μg/ml), aprotinin (2 μg/ml), and 1 mM dithiothreitol (DTT) (600 μl). After centrifugation for 10 minutes at maximum speed in a microfuge, COS cell lysates containing 0.5 to 1 mg of soluble protein were subjected to FPLC on a Mono Q column, and eluted fractions were assayed for MAPK activity (*37*). (**B**) Immunoblot showing expression of MEKK in cells transfected with vector only (control) or vector encoding MEKK that were treated with or without EGF. Equal amounts (100 μg) of soluble protein lysate from COS cells were placed on the gel for immunoblotting.

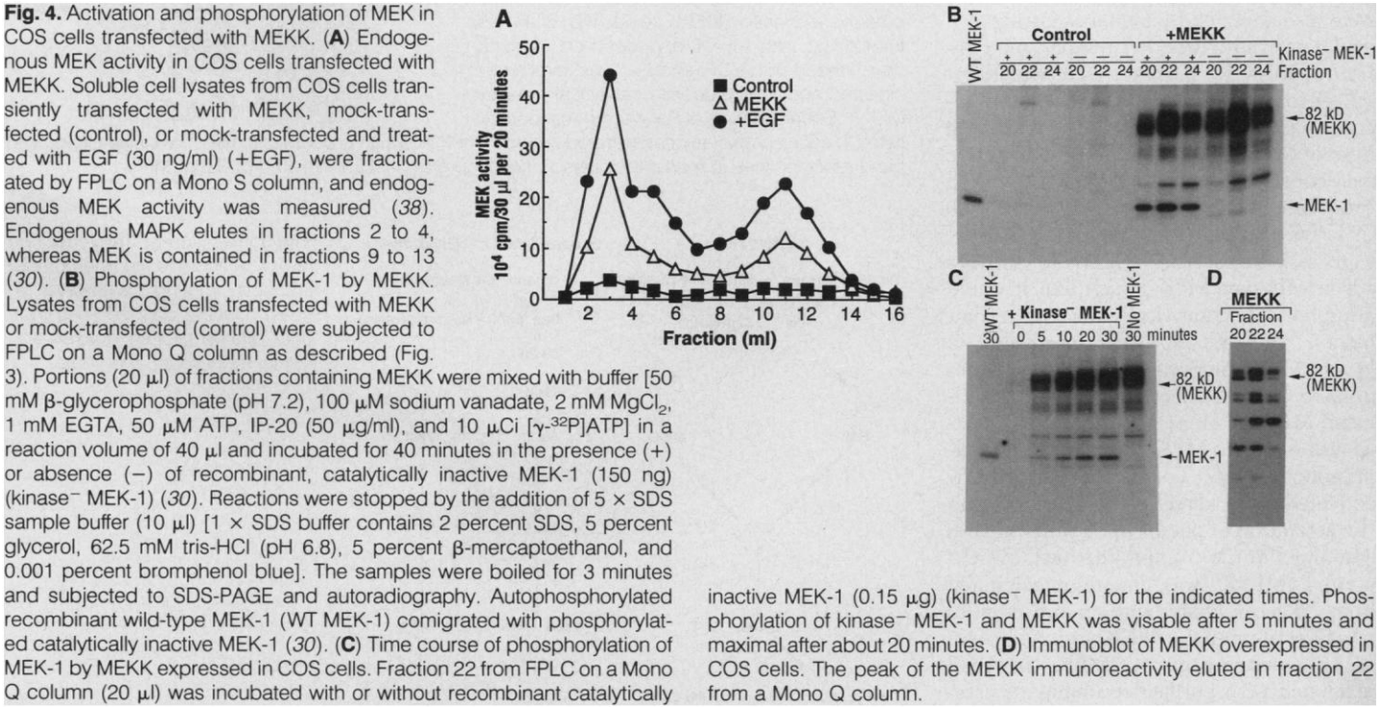

Fig. 4. Activation and phosphorylation of MEK in COS cells transfected with MEKK. (**A**) Endogenous MEK activity in COS cells transfected with MEKK. Soluble cell lysates from COS cells transiently transfected with MEKK, mock-transfected (control), or mock-transfected and treated with EGF (30 ng/ml) (+EGF), were fractionated by FPLC on a Mono S column, and endogenous MEK activity was measured (*38*). Endogenous MAPK elutes in fractions 2 to 4, whereas MEK is contained in fractions 9 to 13 (*30*). (**B**) Phosphorylation of MEK-1 by MEKK. Lysates from COS cells transfected with MEKK or mock-transfected (control) were subjected to FPLC on a Mono Q column as described (Fig. 3). Portions (20 μl) of fractions containing MEKK were mixed with buffer [50 mM β-glycerophosphate (pH 7.2), 100 μM sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, 50 μM ATP, IP-20 (50 μg/ml), and 10 μCi [γ-^{32}P]ATP] in a reaction volume of 40 μl and incubated for 40 minutes in the presence (+) or absence (−) of recombinant, catalytically inactive MEK-1 (150 ng) (kinase⁻ MEK-1) (*30*). Reactions were stopped by the addition of 5 × SDS sample buffer (10 μl) [1 × SDS buffer contains 2 percent SDS, 5 percent glycerol, 62.5 mM tris-HCl (pH 6.8), 5 percent β-mercaptoethanol, and 0.001 percent bromphenol blue]. The samples were boiled for 3 minutes and subjected to SDS-PAGE and autoradiography. Autophosphorylated recombinant wild-type MEK-1 (WT MEK-1) comigrated with phosphorylated catalytically inactive MEK-1 (*30*). (**C**) Time course of phosphorylation of MEK-1 by MEKK expressed in COS cells. Fraction 22 from FPLC on a Mono Q column (20 μl) was incubated with or without recombinant catalytically inactive MEK-1 (0.15 μg) (kinase⁻ MEK-1) for the indicated times. Phosphorylation of kinase⁻ MEK-1 and MEKK was visable after 5 minutes and maximal after about 20 minutes. (**D**) Immunoblot of MEKK overexpressed in COS cells. The peak of the MEKK immunoreactivity eluted in fraction 22 from a Mono Q column.

sured by the incorporation of [γ-^{32}P]ATP (adenosine triphosphate) into the MAPK substrate, a peptide derived from the EGF receptor (EGFR). The first peak of activity eluted represents endogenous activated MAPK, which directly phosphorylates the EGFR peptide substrate (*29*). The second peak of activity represented the endogenous MEK in COS cells (*30*). Endogenous MEK was activated in cells overexpressing MEKK; the activity of MEK was approximately half of that observed in control cells stimulated with EGF. Thus, expression of MEKK appears to activate MAPK by activating MEK.

COS cell lysates were fractionated by FPLC on a Mono Q column to partially purify the expressed MEKK (Fig. 4B). Purified recombinant MEK-1 was then used as a substrate for MEKK in the presence of [γ-^{32}P]ATP to determine whether MEKK directly phosphorylates MEK-1. Fractions 20 to 24 of lysate from COS cells transfected with MEKK phosphorylated MEK-1, whereas corresponding fractions of lysates from control cells had little or no MEKK activity. A modified form of MEK-1 that is catalytically inactive was used in the phosphorylation assay to ensure that it did not autophosphorylate as does wild-type MEK-1. Phosphorylation of catalytically inactive MEK-1 by MEKK was time-dependent (Fig. 4C); MEKK was also phosphorylated. The time-dependent increase in MEKK phosphorylation correlated with a decreased mobility of the MEKK protein during SDS-PAGE. Immunoblotting demonstrated that the MEKK protein co-eluted (after FPLC on a Mono Q column) with the peak of activity that phosphorylated MEK (Fig. 4D). The slowly migrating species of MEKK were also detected by immunoblotting.

To determine whether the phosphorylation of MEK by overexpressed MEKK resulted in activation of MEK, we used recombinant wild-type MEK-1 and a modified form of MAPK that is catalytically inactive in a coupled assay system (Fig. 5A). COS cell lysates were separated by Mono Q–FPLC and fractions containing MEKK were assayed for their ability to activate added wild-type MEK-1 such that it would phosphorylate catalytically inactive recombinant MAPK in the presence of [γ-^{32}P]ATP. Fractions 20 to 24 from lysates of COS cells transfected with MEKK activated MEK-1. Thus, MEKK phosphorylated and activated MEK-1, leading to MAPK phosphorylation. To ensure that MEKK activated MEK directly, and not through the activation of one or more other kinases contained in the column fractions, overexpressed MEKK was immunoprecipitated from COS cell lysates with an antiserum to a COOH-terminal MEKK fusion protein (*31*). Immunoprecipitated MEKK was then incubated with purified recombinant catalytically inactive MEK-1 in the presence of [γ-^{32}P]ATP (Fig. 5B). MEKK phosphorylated catalytically inactive MEK-1, which comigrated with wild-type MEK-1 on SDS-PAGE. Several phosphorylated bands of overexpressed MEKK were detected in the

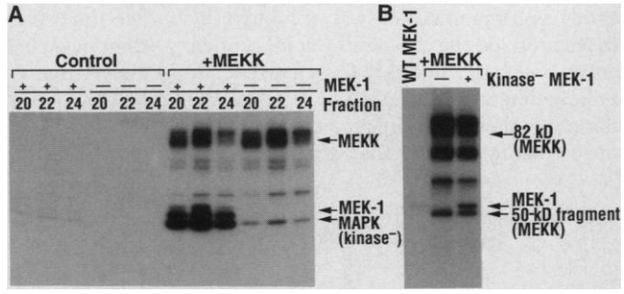

Fig. 5. Activation of MEK by MEKK. (**A**) Phosphorylation of MAPK by activated MEK-1. Lysates from COS cells transfected with MEKK or mock-transfected (control) were fractionated by FPLC on a Mono Q column and portions (20 μl) of fractions containing MEKK (20, 22, and 24) were mixed with buffer as described (Fig. 4). Each fraction was incubated in the presence (+) or absence (−) of purified recombinant wild-type MEK-1 (150 ng) and in the presence of purified recombinant, catalytically inactive (kinase⁻) MAPK (300 ng). Phosphorylation of recombinant wild-type MEK-1 by MEKK enhanced the phosphorylation of catalytically inactive MAPK. MEKK did not phosphorylate MAPK in the absence of added recombinant MEK-1. (**B**) Phosphorylation of MEK-1 by immunoprecipitated MEKK. MEKK was immunoprecipitated from lysates of COS cells overexpressing MEKK with an antiserum to a fusion protein containing the COOH-terminal portion of MEKK (*31*). Immunoprecipitated MEKK was resuspended in 10 to 15 μl of PAN [10 mM piperazine-*N*,*N*′-bis-2-ethanesulfonic acid (Pipes) (pH 7.0), 100 mM NaCl, and aprotinin (20 μg/ml)] and incubated with (+) or without (−) catalytically inactive MEK-1 (150 ng) and 25 μCi of [γ-^{32}P]ATP in buffer [20 mM Pipes (pH 7.0), 10 mM $MnCl_2$, and aprotinin (20 μg/ml)] in a final volume of 20 μl for 15 minutes at 30°C. Reactions were stopped by the addition of 5× SDS sample buffer (5 μl). The samples were boiled for 3 minutes and subjected to SDS-PAGE and autoradiography as described (Fig. 3).

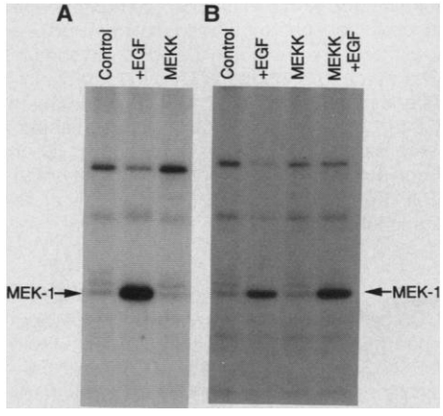

Fig. 6. Activity of Raf in COS cells overexpressing MEKK. (**A**) Phosphorylation of MEK-1 by activated Raf. COS cells were transiently transfected with vector alone (control) or with the pCVM5-MEKK construct (MEKK) as described (Fig. 3). Quiescent control cells were treated with or without human EGF (30 ng/ml) for 10 minutes and Raf was immunoprecipitated from cell lysates with an antibody to a COOH-terminal peptide from Raf (*32*). Immunoprecipitated Raf was incubated with catalytically inactive MEK-1 (150 ng) and 25 μCi of [γ-^{32}P]ATP as described (Fig. 5). (**B**) Activity of Raf in COS cells overexpressing MEKK treated with EGF. Cells transfected with MEKK and deprived of serum were treated with EGF, and Raf was immunoprecipitated and incubated with catalytically inactive MEK-1. Equal amounts of Raf were immunoprecipitated in each sample as demonstrated by immunoblotting with antibodies to Raf. The slowest migrating band represents an immunoprecipitated phosphoprotein that is unrelated to Raf or MEK-1.

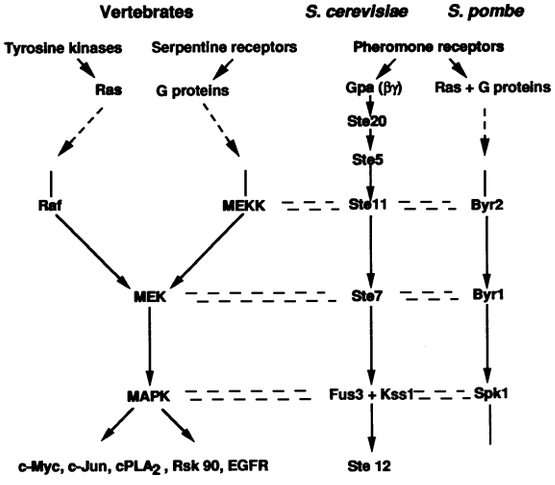

Fig. 7. Relation of the vertebrate MAPK network to the pheromone-induced mating pathways in yeast. Double dashed lines indicate related protein kinases acting in vertebrates and yeast.

immunoprecipitates. These bands probably resulted from autophosphorylation of MEKK and corresponded to the forms of MEKK identified by immunoblotting of lysates from COS cells transfected with MEKK (Fig. 2C). Immunoprecipitates obtained with pre-immune serum contained no MEKK and did not phosphorylate MEK-1. Thus, MEKK appears to directly phosphorylate MEK, although an associated kinase that co-immunoprecipitates with MEKK cannot be unequivocally excluded.

Our results show that MEKK can phosphorylate and activate MEK, which in turn phosphorylates and activates MAPK. Raf can also phosphorylate and activate MEK (*21*, *22*) (Fig. 6). COS cells deprived of serum were stimulated with EGF, and Raf was immunoprecipitated with an antibody to the COOH-terminus of Raf-1 (*32*). The immunoprecipitated Raf phosphorylated MEK-1 in the presence of [γ-^{32}P]ATP (Fig. 6A). Little or no phosphorylation of MEK-1 by Raf was observed in immunoprecipitates from untreated control cells. Overexpression of MEKK did not activate Raf, even though MAPK and MEK were activated (Figs. 3A and 4A). Activated MEKK was not present in immunoprecipitates of Raf from COS cells overexpressing MEKK. Treatment of COS control cells or COS cells overexpressing MEKK with EGF resulted in a similar degree of phosphorylation of MEK-1 by immunoprecipitated Raf (Fig. 6B). The amount of Raf in the immunoprecipitates from control cells and cells transfected with MEKK was similar as shown by subsequent SDS-PAGE and immunoblotting with the antibody to Raf (*27*). Thus, both MEKK and Raf can independently activate MEK.

Our identification of the MEKK from mouse further substantiates the conservation in regulation of the MAPK system between yeast and mammals. The results indicate that the mammalian regulatory network controlling MAPK is more complicated than that in yeast. Both MEKK and Raf activate MEK; this appears to be a convergence point immediately upstream of MAPK for various signals from the cell surface. Raf may regulate the MAPK network primarily in response to receptors that have associated tyrosine kinase activity, whereas MEKK might mediate primarily signals originating from receptors that activate G proteins and protein kinase C (Fig. 7). This possibility is supported by the findings in specific cell types that down-regulation of protein kinase C does not inhibit activation of MAPK in response growth factors, but does inhibit activation of MAPK in response to agents that stimulate the muscarinic M_1 receptor (*1*, *11*, *33*). The demonstration that Ste20, a protein kinase in *S. cerevisiae*, is upstream of Ste11 in the pheromone-response pathway further supports this hypothesis (*34*). In PC12 cells, expression of dominant negative mutant Ras does not inhibit activation of MAPK in response to stimulation of G protein–coupled receptors or activation of protein kinase C (*11*, *35*), but does inhibit activation of MAPK in response to NGF or FGF (*9*, *10*). However, this hypothesis may be too simplistic, and more complex, cell type specific roles of Raf and MEKK in integrating tyrosine kinase and G protein–coupled signaling may exist. Defining MEKK and Raf as a divergence in the MAPK network provides a mechanism for differential regulation of this system.

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28. A cDNA encoding p42 MAPK from *Xenopus laevis* was cloned into the pRSETB expression vector (Invitrogen). This construct was used for expression in the LysS strain of *Escherichia coli* BL21(DE3) of a p42 MAPK fusion protein containing a polyhistidine sequence at the NH_2-terminus. Cultures containing the expression plasmid were grown at 37°C to an optical density of 0.7 to 0.9 at 600 nM. Isopropyl-β-thiogalactopyranoside (0.5 mM) was added to induce fusion protein synthesis and the cultures were incubated for 3 hours. The cells were then collected and lysed by freezing, thawing, and sonication. The lysate was centrifuged at 10,000*g* for 15 minutes at 4°C. The supernatant was then passed over a Ni^{2+}-charged Sepharose resin (Qiagen) according to the manufacturer's directions and the soluble recombinant MAPK was eluted in sodium phosphate buffer (pH 4.5). The purified recombinant MAPK was more than 80 percent pure. The purified recombinant MAPK served as a substrate for MEK and catalyzed the phosphorylation of a peptide consisting of residues 662 to 681 of the EGF receptor ($EGFR^{662–681}$).
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30. A cDNA encoding MEK-1 was obtained from mouse B cell cDNA templates with the polymerase chain reaction and oligodeoxynucleotide primers corresponding to portions of the 5′ coding region and 3′ untranslated region of the reported sequence (*17*). The catalytically inactive MEK-1 was generated by site-directed mutagenesis of Lys^{343} to Met. The wild-type MEK-1 and catalytically inactive MEK-1 proteins were expressed in pRSETA as recombinant fusion proteins containing a polyhistidine sequence at their NH_2-termini, as described (*28*). Endogenous MEK activity was characterized by fractionation on Mono S FPLC (A. M. Gardner, in preparation).
31. The MEKK cDNA was digested with Pst I and Kpn I, thereby creating a 1670-bp fragment that encodes the catalytic domain of MEKK. This fragment was expressed in pRSETC as a recombinant fusion protein containing a polyhistidine sequence at its NH_2-terminus and purified as described (*28*). The purified COOH-terminal MEKK fusion protein was used to generate polyclonal antisera.
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38. For assaying endogenous MEK activity, cells were washed twice in cold PBS and lysed in 650 μl of a solution containing 50 mM β-glycerophosphate, 10 mM 2-[*N*-morpholino]ethane sulfonic acid (pH 6.0), 100 μM sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, Triton X-100 (0.5 percent), leupeptin (5 μg/ml), aprotinin (2 μg/ml), and 1 mM DTT. After centrifugation at maximum speed for 10 minutes in a microfuge, soluble cell lysates (1 to 2 mg of protein) were applied to a Mono S column equilibrated in elution buffer [50 mM β-glycerophosphate, 10 mM MES (pH 6.0), 100 μM sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT]. The column was washed with buffer (2 ml) and bound proteins were eluted with a 30-ml linear gradient of 0 to 350 mM NaCl in elution buffer. A portion (30 μl) of each fraction was assayed for MEK activity by mixing with buffer [25 mM β-glycerophosphate, 40 mM Hepes (pH 7.2), 50 μM sodium vanadate, 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (3000 to 4000 cpm/pmol), inhibitor protein–20 (IP-20; TTYADFIASGRTGRRNAIHD; 25 μg/ml), 0.5 mM EGTA, recombinant MAP kinase (7.5 μg/ml) (*28*), and 200 μM $EGFR^{662–681}$] in a final volume of 40 μl. After incubation at 30°C for 20 minutes, the incorporation of [γ-^{32}P]ATP into $EGFR^{662–681}$ was measured as described (*37*).

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